BioMetals 1996, 9, 245–248

Spectrophotometric competition study between molybdate and Fe(III) hydroxide on *N*,*N'*-bis(2,3-dihydroxybenzoyl)-L-lysine, a naturally occurring siderophore synthesized by *Azotobacter vinelandii*

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Received 24 October 1995; accepted for publication 2 January 1996

The solubilization of Fe(III) hydroxide by the naturally occurring siderophore *N*,*N'*-bis(2,3-dihydroxybenzoyl)-L-lysine has been investigated spectrophotometrically in the presence and the absence of a stoichiometric amount of molybdate in aqueous medium at pH 7. In the absence of molybdate the reaction is 50% complete after 115 min. In contrast, the addition of an equimolar amount of molybdate results in an instantaneous formation of the molybdenum siderophore complex and a significant delay in the formation of the corresponding iron complex: 50% of the iron complex is present after 44 h and equilibrium is only reached after 2 weeks. The results are discussed with regard to metal acquisition by the nitrogen fixing cells of *Azotobacter vinelandii*.

Keywords: siderophore-promoted dissolution, iron complexes, molybdenum complexes, metal uptake

Introduction

Iron as well as molybdenum is a component of the conventional nitrogenase, and both metals are essential for the optimal growth of *Azotobacter vinelandii*. Molybdenum occurs in oxidizing conditions and in dilute solutions in the environment as water soluble monomeric molybdate whereas Fe(III) hydrolyzes to form highly insoluble ferric hydroxides and oxides ($K_{\mathrm{sol.}} = 10^{-37}$ to 10^{-44}) (Schwertmann 1991, Da Silva & Williams 1992). The solubility and the dissolution rate of iron hydroxides and oxides is essential for the iron supply to microorganisms. Dissolution can take place by protonation, reduction and complexation. Ligand-promoted dissolution reactions of Fe(III) oxides have been investigated (Schwertmann 1991, Hersman *et al.* 1995) and are determined by solution variables (e.g. reactant concentrations, pH) as well as solid phase variables (type of iron source, surface area, crystallinity).

It is well established that microorganisms excrete low-molecular weight chelators, so called siderophores, in response to iron deficiency to solubilize Fe(III) for transport into the cell (Hider 1984, Raymond 1984). Although molybdenum deficiency can also limit the nitrogen fixing process (Pienkos *et al.* 1977), the uptake and transport of this element is still less well understood. A recent mutational study of genes involved in molybdenum transport in *A. vinelandii* provided evidence for a specific high- and low-affinity molybdenum transport system, and the possibility that siderophores might be involved in the low-affinity molybdenum uptake system that functions at concentrations above 10 μM molybdenum (Mouncey *et al.* 1995).

Most siderophores are hexadentate ligands which form six coordinate octahedral complexes of high stability with iron. In contrast the N_2-fixing cells of *A. vinelandii* produce *N*,*N'*-(2,3-dihydroxybenzoyl)-L-lysine (LYSCAM; Figure 1) (Corbin & Bulen 1969), a bis(catecholamide) siderophore, ideal for the complexation of the MoO_2^{2+} unit, which has four vacant coordination sites.

Under normal environmental conditions both metals compete for the siderophore ligand. Complex formation is governed by the thermodynamic stabilities of the complexes but also controlled by kinetic factors. Interestingly, the molybdenum accumulation of *A. vinelandii* is very rapid. It has been reported that the nitrogen-fixing cells are able to exhaust the growth medium of molybdate within 1 h (Pienkos & Brill 1981). With this in mind we have investigated the competitive complex formation of molybdate and iron hydroxide for LYSCAM in aqueous medium at pH 7.

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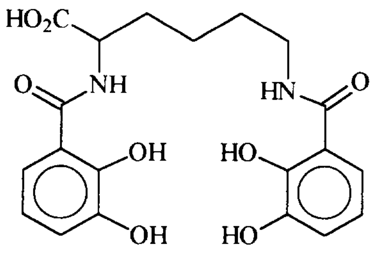

Figure 1. LYSCAM.

Materials and methods

For our investigations we used freshly prepared iron hydroxide. Due to its high surface area and structural disorder it dissolves much faster than, for example, hematite or goethite and therefore it should be the iron source that competes most effectively with molybdate for the siderophore ligand. The initial siderophore concentration used was 0.4 mM, which lies within the geochemically significant range estimated for soil bacteria (Hersman *et al.* 1995).

All solutions were made up with 18 MΩ cm^{-1} water from a Millipore Milli-Q system. $K_2MoO_4 \cdot 2H_2O$ (Fluka purum, Gillingham, UK) and HEPES potassium salt (Sigma, Poole, UK) were used as commercially supplied. Stock solutions were prepared by dissolving weighed amounts of the reagents in deionized water. Iron was introduced into the solutions by using an iron standard solution for AAS (Aldrich, Gillingham, UK). LYSCAM was synthesized according to a literature procedure (Chimiak & Neilands 1984).

The electronic absorption spectra were recorded on a Perkin-Elmer Lambda 5 UV-vis spectrophotometer, equipped with a PC for data collection and evaluation. The temperature for the study was maintained at 25±0.5°C. To precipitate ferric hydroxide the pH value of a solution of 6 μmol Fe(III) in 10 ml 0.1 M HEPES buffer was adjusted to pH 7 with concentrated HCl and carbonate-free KOH in a jacketed thermostated titration vessel. After 30 min, 60 μl of a 0.1 M K_2MoO_4 solution followed by a solution of 2.52 mg (6 μmol) LYSCAM in 5 ml HEPES buffer (pH 7) were added, resulting in a mixture 0.4 mM each in molybdate, total iron and ligand. The time of mixing was recorded as zero time and the first scans obtained after 33 min. A further 19 spectra were recorded at periodic intervals of 30 min. A peristaltic pump was used to circulate the reaction mixture from the vessel through a filter probe to a flow-through cuvette of 1 cm optical pathlength at a flow rate of 1.25 ml min^{-1}. The pH value was checked throughout the experiments and it remained at 7.00±0.02. Control experiments were carried out analogously in the absence of molybdate. In these cases 20 spectra were taken in intervals of 10 min. Further spectra were recorded at rather long intervals until there was no further change with time. All dissolution experiments were done in triplicate and the suspensions were stirred throughout the whole experiments.

Results and discussion

Solubilization of iron hydroxide by LYSCAM

Figure 2 shows the spectral changes observed at the beginning of the solubilization reaction of Fe(III) hydroxide in the presence of LYSCAM at pH 7. The maximum of the broad charge transfer band of the iron siderophore complex is not only increasing but also shifting slowly from 500 nm to higher wavelengths. At the beginning of the solubilization the ligand is in excess over free Fe(III) and the high affinity of catecholate groups for iron leads predominantly to the formation of tris(catecholato) species, showing a characteristic maximum absorbance between 480 and 495 nm (Harris *et al.* 1981). In the course of the reaction the ratio of ligand to iron decreases and the concentration of bis(catecholato) complex increases, leading to the shift of the maximum to higher wavelengths. As shown in Figure 3(a), equilibrium is attained after 2 days, with a maximum absorbance at 562 nm (Figure 4a), characteristic of Fe(III) bis(catecholato) complexes (Harris *et al.* 1981). This is consistent with the equimolar concentration of Fe(III) and bis(catecholamide) ligand, achieved after complete dissolution of the iron hydroxide.

As the environment, our model system is not homogeneous, equilibrium has to be established between the water soluble ligand, the metal complexes and the suspended iron hydroxide particles. The dimensions of these particles and their degree of crystallinity influence their activity. Therefore we precipitated the iron hydroxide under defined and reproducible conditions each time 30 min before we started the experiment. As shown in Figure 3(a), LYSCAM removes approximately 50% of the Fe(III) from the suspended solid within 115 min. Compared with the hexadentate hydroxamate siderophore deferriferrichrome A, that removes Fe(III)

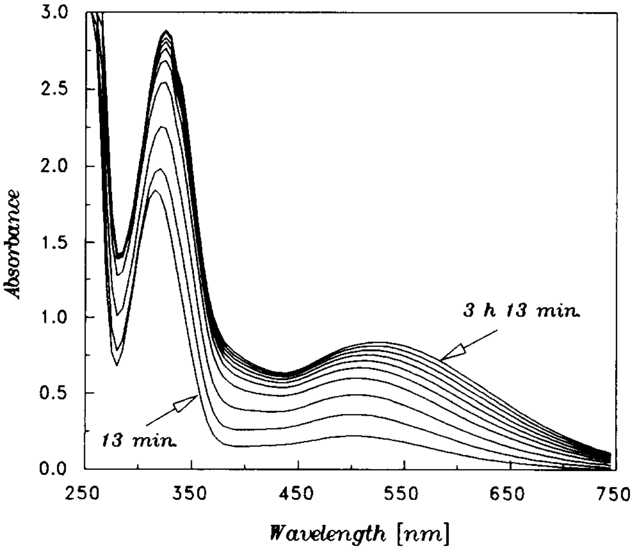

Figure 2. UV-vis spectral changes accompanying the solubilization of Fe(III) hydroxide by LYSCAM at pH 7, recorded at periodic intervals of 20 min.

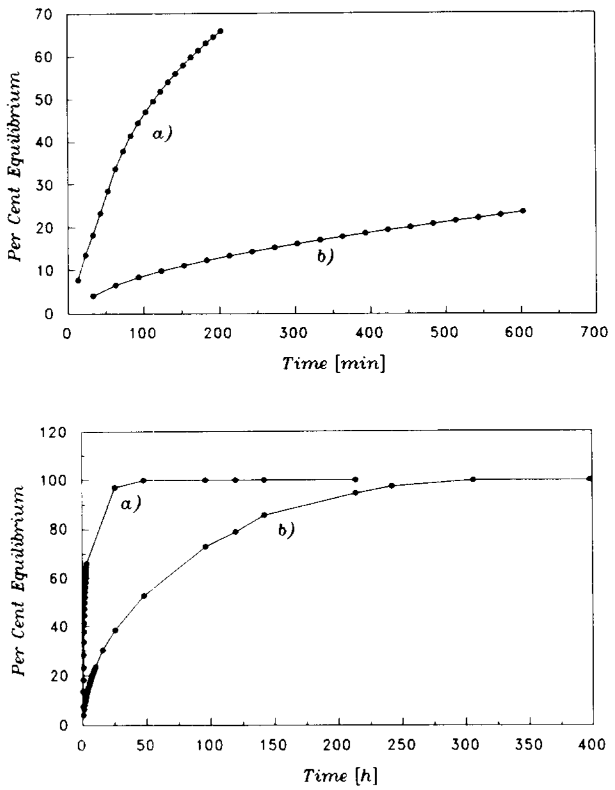

Figure 3. The time course of the solubilization of Fe(III) hydroxide by LYSCAM (**a**) in the absence and (**b**) in the presence of an equimolar amount of molybdate, shown at two different time scales. The per cent equilibrium is based on the absorbance at 562 nm when equilibrium was reached.

from iron hydroxide with a half-life of 30 min (Emery 1978), LYSCAM is the slightly less efficient iron chelator, probably due to its lower denticity.

Solubilization of iron hydroxide in the presence of molybdate

In the presence of an equimolar amount of molybdate, the formation of the iron siderophore complex is significantly delayed. In contrast, the formation of the molybdenum LYSCAM complex is essentially complete within the time of mixing. The orange molybdenum complex shows a maximum of absorbance at 385 nm. As seen in Figure 5, the absorbance between 385 and 450 nm is decreasing slowly while the absorbance around 562 nm increases, due to the formation of iron complex.

The exchange reaction is extremely slow: 50% of the equilibrium concentration of iron complex was observed after 44 h (Figure 3b) and equilibrium is reached only after weeks. The equilibrium spectra of both experiments shown in Figure 4 are very similar, demonstrating that the iron complex is evidently the thermodynamically favoured one towards the kinetically controlled reaction moves.

The slow metal exchange observed for this system is in marked contrast with the usual substitutional lability of high-spin d^5 Fe(III) and d^0 Mo(VI) coordination compounds, but consistent with the observed slow iron exchange between two different siderophores (Tufano & Raymond 1981). The reason is essentially the same in both cases: the exchange between siderophore metal complexes in which the denticity of the ligand matches the coordination number of the metal requires partial unwrapping of the multidentate ligand prior to attack of the competing species. In our case the competing species could be either $Fe(III)_{aq.}$ or the surface of the iron hydroxide particles.

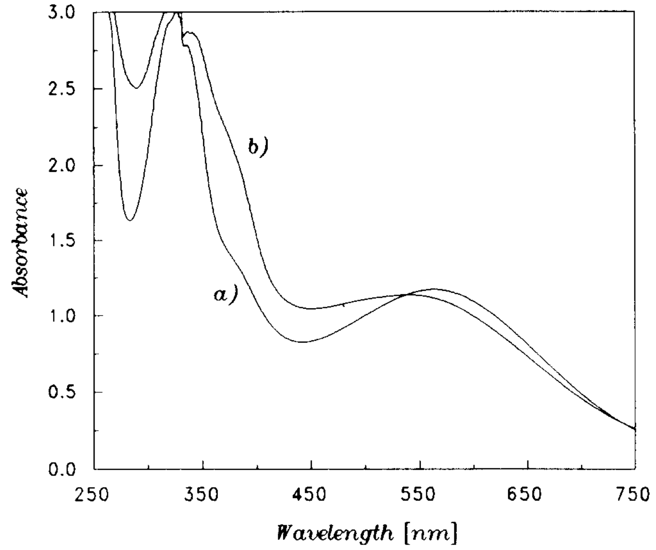

Figure 4. Equilibrium spectra measured after solubilization of Fe(III) hydroxide by LYSCAM (**a**) in the absence and (**b**) in the presence of molybdate, recorded after 2 days and 2 weeks, respectively.

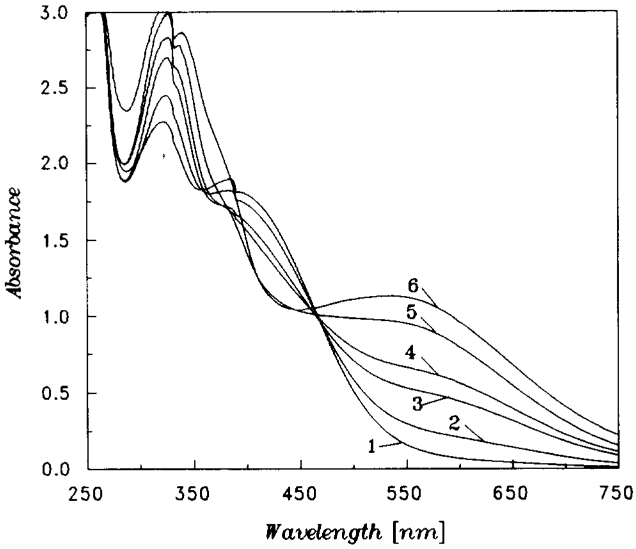

Figure 5. UV-vis spectral changes accompanying the solubilization of Fe(III) hydroxide by LYSCAM in the presence of an equimolar amount of molybdate at pH 7, recorded after 1, 33 min; 2, 10 h; 3, 1 day; 4, 2 days; 5, 6 days; 6, 2 weeks.

During the exchange reaction the formation of heterometallic complexes of the kind $[FeMoO_2(LYSCAM)_2]^{5-}$ is conceivable, as the molybdenum complex could be present as monomeric as well as dimeric species in solution. Model complexes for both species have recently been characterized by X-ray crystallography (Albrecht *et al.* 1994, Duhme *et al.* 1996).

Our observations elucidate the complexity of structural, thermodynamic and kinetic factors regulating the solubilization process in the model system. Under environmental conditions, however, the situation is even more complicated as a variety of metal ions compete for a large number of ligands. Although equilibrium concentrations are determined by the magnitude of the relative stability constants, as long as the system is not at equilibrium—and the environment represents a system in dynamic flux—concentrations are controlled by kinetic limitations.

In this context the observed extreme delay of iron solubilization in the presence of molybdate is of importance, especially because molybdenum uptake by *A. vinelandii* has been reported to be a rapid process. Although the iron complex of LYSCAM is thermodynamically more stable, its formation in aqueous solution at pH 7 is limited by the low concentration of available Fe(III) due to the insolubility of iron hydroxide. In contrast the corresponding molybdenum complex is formed quantitatively within seconds and appears to be sufficiently inert, at least in this model system, for potential accumulation by microorganisms.

Acknowledgment

A.K.D. gratefully acknowledges a DFG postdoctoral fellowship.

Note added in proof

We would also like to draw the attention of the reader to the following recent publication: Cornish AS, page WJ. 1995 Production of the triacetecholate siderophore protochelin by *Azotobacter vinelandii*. *BioMetals* **8**, 332–338

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